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MOLECULAR AND CELLULAR BIOLOGY, Jan. 1995, p. 540–551
0270-7306/95/$04.00+0

Vol. 15, No. 1

Sophia

Selection of Efficient Cleavage Sites in Target RNAs by Using a Ribozyme Expression Library

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Received 21 July 1994/Returned for modification 13 September 1994/Accepted 12 October 1994

Inactivation of gene expression by antisense mechanisms in general and by ribozymes in particular is a powerful technique for studying the function of a gene product. We have designed a strategy for expression of ribozymes, for selection of accessible cleavage sites in target RNAs, and for isolation of ribozymes from a library of random sequences flanking the unique sequence of a hammerhead. The expression cassette for ribozyme genes is based on adenovirus-associated RNA. Alternatively, we used polymerase III or the T7 phage transcription machinery. The ribozyme sequences are positioned in the center of a stable stem-loop structure, allowing for a correctly folded ribozyme region within the expressed RNA. A library of ribozyme genes with random sequences of 13 nucleotides on both sides of the hammerhead was generated. As an example, ribozymes which are specific for seven sites within the mRNA or nuclear RNA of human growth hormone were selected and identified. Sequencing of ribozyme genes reamplified from the library confirmed not only the predicted cleavage sites but also the presence of different ribozyme variants in the library. In a test of the ribozyme variants for repression of growth hormone synthesis in a cellular assay, the strongest effect (more than 99% inhibition) was found for the variant with the shortest stretch of complementarity (7 and 8 nucleotides on either side) to the target RNA. This basic strategy seems to be applicable to the selection of suitable target sites and to the isolation of corresponding ribozymes for any mRNA of interest.

Inactivation of gene function by reverse genetics is very important for elucidating the function of a particular gene and could also have a great impact on the treatment of infectious and other diseases based on aberrant gene expression. Gene function can be inactivated by homologous recombination at the DNA level, by antisense nucleic acids or ribozymes at the RNA level, or by antibodies at the protein level. All four approaches have advantages and disadvantages. For therapeutic application, only targeting of RNA by antisense molecules or ribozymes seems to be feasible. Both classes of molecules can be generated by chemical synthesis or, when linked to a promoter, by transcription in vitro or even in vivo. The principle of catalytic self-cleavage of RNA molecules (9) and of cleavage in *trans* (2) has been well established within the last 10 years. Among the RNA molecules with ribozyme activity, the hammerhead ribozymes are the best characterized (43). After it was shown that the hammerhead structure can be integrated into a heterologous RNA sequence and thereby confer ribozyme activity to this molecule (20), it seemed obvious that catalytic antisense sequences could be designed for almost any target sequence containing the consensus cleavage site GUC (15, 22, 35). The basic principle of ribozyme design seems to be rather simple: select a region in the RNA of interest which contains the triplet GUC (or, in general, NUH [where H is A, C, or T]), create two stretches of antisense oligonucleotides 6 to 8 nucleotides (nt) long, and put the sequence forming the catalytic hammerhead in between. Molecules of this kind have been synthesized for various target sequences and have been shown to have catalytic activity in vitro and in some cases also in vivo. The actual challenge for in vivo application was the construction of ribozyme genes which would allow for continuous expression of the ribozyme in a particular cell. However, targeted cleavage of mRNA by ribozymes in vivo has proven to be difficult to demonstrate (12, 48) and has been shown in only three cases (8, 37, 40). Nevertheless, a substantial biological effect of some ribozyme constructs was observed in vivo in some cases (41, 49). Similar biological effects could also be exerted by the antisense sequence only (37, 40). Despite an increasing number of successful studies based on general rules for antisense sequences and on sophisticated constructions for the expression of ribozymes, the generation of ribozymes which cleave RNA in vivo is still a matter of trial and error.

There are at least five potential reasons for the limited ability of expressed ribozymes to function satisfactorily within the complex cellular milieu. (i) Within the cell, the substrate mRNA is presumably in a highly folded structure and may also be protected by proteins bound to part of the molecule. The targeting to accessible sites within the substrate for hybridization with the complementary flanking regions of a ribozyme is currently a matter of chance (21, 40). Computer-assisted prediction of possible secondary structures which are thermodynamically stable may be helpful in the search for loop regions without base pairing, but the physiological relevance of these conformational models is still uncertain. (ii) The spliced mRNA is rapidly removed from the nucleus (12). The ribozyme should also be transported to the cytoplasm (to the same subcellular compartment) with the mRNA, preferably by the same pathway. A strategy for colocalization of human immunodeficiency virus ribozymes with their substrate has recently been described (41). The use of packaging signals of RNA viruses could be feasible for the development of certain antiviral ribozymes. (iii) The application of ribozymes in vivo requires the insertion of ribozyme genes in suitable expression cassettes. Transcription of these constructs could produce

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RNAs for which the central catalytic secondary structure of the ribozyme is abolished by other, more stable intramolecular base pairings within the vector-derived noncomplementary flanking sequences (4, 14, 34). (iv) An excess (100- to 1,000-fold) of ribozyme molecules over target transcripts seems to be required to achieve a detectable decrease in mRNA levels (12, 45). The production of 10^5 to 10^6 ribozyme molecules per cell over a long period could have cytotoxic effects. Generally, such high levels of expression are not stable over a longer period (29, 47). The actual requirement of this large excess of ribozymes could be due to insufficient stability of ribozymes against nucleases or to inefficient transport to the cytoplasm. (v) The kinetics of the cleavage reaction and the ability of ribozymes to carry out multiturnover reactions depend on the binding parameters and the structure of the complementary flanking regions of the ribozyme. It seems that cellular proteins can influence the catalysis of the cleavage reaction, probably by aiding the dissociation of the ribozyme from the cleaved substrate, which is a prerequisite for the next round of cleavage (6, 42). It is impossible to predict a priori the optimal structure of flanking regions of a ribozyme which would allow for a high specificity and for multiple turnover reactions (17, 21, 22).

Several of the points raised above have been addressed in a recent paper by Bertrand et al. (5). The authors concluded from their data that the specificity and efficiency of the ribozyme are dependent on several parameters which affect ribozyme-target hybridization. That paper confirmed that finding the target sequence which is accessible in vivo is the most difficult task in designing a suitable ribozyme. It is therefore tempting to design a system in which the most suitable ribozyme is selected by the target sequence itself from a pool of ribozyme molecules with known stability and structure that differ only in the actual antisense part of the sequence. We have developed such a system. The strategy is largely dependent on the function of an optimized expression unit allowing for high-level expression and a stable structure of the ribozyme. It is based on a representative library of random sequences flanking a hammerhead structure embedded in a stable loop structure within an adenovirus-associated RNA (vaRNA). Here we present data which clearly show that our ribozyme expression library contains ribozymes for various target genes. The target-selected ribozymes function efficiently in vivo.

MATERIALS AND METHODS

Construction of the ribozyme expression vector. Oligonucleotides were synthesized in an automatic synthesizer (Applied Biosystems). The genes for vaRNAs I and II were cloned as an *Xba*I-*Nsi*I fragment (1) in the *Xba*I-*Pst*I site of pGEM7zf+ after prior deletion of the *Aat*II site in pGEM. The resulting plasmid was named pGva. Oligonucleotides I (5'CGTCGACTGCTGCAGTG CAGCGTGTGGACCCAACGACACGCGGGCGGTAACCGACGT3') and II (5'CGGTTACCGCCCGCGTGTCGTTGGGTCCACACGCTGCACTGCAG CAGTCGACGACGT3') (10 ng each), which represent both strands of the sequence which should form a loop, were annealed in 20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$ by heating for 5 min at 85°C and slow cooling to room temperature and cloned into the *Aat*II site within the va gene sequence. The resulting plasmid was designated pGvaL (see Fig. 1). The 5' end of oligonucleotides III [5'CCGCTCGAG$(N)_{13}$CTGATGAGTCCGTGAGGACGAAA3'] and IV [5'TGCATGCAT$(N)_{11}N_G$TTTCGTCCTCACGGACTCATCAG3', where N_G is 40% C, 40% G, 10% T, and 10% A] for the randomly mutated ribozymes were phosphorylated with polynucleotide kinase.

Oligonucleotides III and IV (5 μg each) were heated for 5 min at 85°C in 20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$, cooled to 65°C, and incubated with 200 μM deoxynucleoside triphosphates (dNTPs) 2.5 U of *Taq* polymerase for 30 min at 65°C. After phenol extraction and ethanol precipitation, the double-stranded oligonucleotides were digested with *Nsi*I-*Xho*I overnight and cloned into the *Sal*I and *Pst*I sites of pGvaL. Ligation products were transformed in highly competent (10^{10} colonies per μg of DNA) *Escherichia coli* DH5, and plasmid DNA from a pool of 10^9 different individual clones was prepared. The ribozyme gene library was designated GvaLRz. The corresponding RNAs were called va, vaL, and vaLRz.

Plasmid pMTT7N contains the gene for T7 phage RNA polymerase, modified at the 5' end with the nuclear localization signal of simian virus 40 T antigen under the control of the mouse metallothionein promoter (28).

pol III-dependent in vitro transcription with HeLa extracts. Whole-cell HeLa extracts were prepared by the method described previously (19, 29). Transcription assays were performed by incubating 1 μg of linear or circular DNA template, 5 μl of HeLa extract (15 mg of total protein per ml in a mixture of 20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M KCl, 20% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride), 3 μl of 1.0 M KCl, 2.5 μl of 0.1 M $MgCl_2$, 1.0 μl of 1.0 M *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES; pH 7.9), 2.5 μl of 10 mM dithiothreitol, 5 μl of 5 mM NTPs (plus 10 μCi of [^{32}P]CTP), and 10 U of RNasin in a total volume of 50 μl at 30°C for 90 min. After digestion with 23 U of DNase I at 30°C for 15 min, the reaction products were purified by three phenol extraction procedures, ethanol precipitated, and electrophoretically separated on 6% denaturing polyacrylamide (PAA) gels. Usually, 80 to 100 ng of vaRNA per 50-μl reaction volume was synthesized.

For preparative ribozyme synthesis by polymerase III (pol III), the in vitro transcription was performed in a 20× (1-ml) reaction mixture and the purification was carried out with glasmilk (RNaid Kit; Bio 101, La Jolla, Calif.) after preparative electrophoresis on a 3% native NuSieve agarose gel.

T7 polymerase-dependent in vitro transcription. Transcription in vitro was performed by incubating 2 μg of DNA template, 12.5 μl of TKB (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M KCl, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride), 10 U of RNasin, 2.5 μl of 5 mM NTPs (or 5 mM ATP, GTP, and TTP plus 20 μCi of [^{32}P]CTP and 1 mM CTP), 2.5 μl of 10 mM dithiothreitol, 100 U of T7 RNA polymerase (Biolabs) in a total volume of 25 μl at 37°C for 60 min. After digestion with 23 U of DNase I, the transcripts were purified twice by phenol extraction and ethanol precipitated.

The amount of RNA synthesized was estimated after gel electrophoresis in an ethidium bromide-stained agarose gel calibrated with concentration markers. In a standard reaction, 5 to 8 μg of RNA per 25-μl reaction volume was synthesized.

Cleavage reaction with ribozymes in vitro. For analytical analysis, 100 nM ribozyme and 100 nM in vitro-transcribed substrate human growth hormone (hGH) RNA were mixed in a 15-μl reaction volume containing 50 mM Tris (pH 7.5) and 1 mM EDTA. For heat denaturation, the mixture was boiled at 95°C for 90 s and quickly cooled on ice. $MgCl_2$ (10 mM) was added, and the mixture was incubated at 37°C for 30 or 60 min. The reaction was stopped by addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol), the mixture was heated at 95°C for 2 min, and the products were analyzed in a 4 or 6% polyacrylamide–8 M urea gel in Tris-borate EDTA buffer.

Preparation of cellular RNA. Cytoplasmic RNA was prepared by Nonidet P-40 lysis and nuclear separation by the method of Chen-Kiang and Lavery (10). Total-cell RNA was extracted by the guanidinium-phenol method (11) or with the RNaid Plus Kit (Bio 101).

Northern blot analysis. RNA samples were fractionated on a 1.5% agarose gel containing 2.2 M formamide and transferred to Hybond N+ nylon membrane. Northern (RNA) hybridization was carried out with ^{32}P-labelled probes by the method described by Westneat et al. (46). hGH and vaDNA sequences were labelled by standard random-priming techniques with DNA pol I (Klenow fragment). The same amounts of total RNA or cytoplasmic RNA were applied in all lanes of the gels.

Cleavage of cellular RNA in vitro by ribozymes from the library. Purified total-cell RNA and a cytoplasmic RNA-protein fraction were used as the source of mRNA. For preparing the cytoplasmic RNA fraction, 10^5 cells were washed with phosphate-buffered saline, resuspended in 50 mM Tris-HCl (pH 7.5), incubated on ice for 10 min, and lysed by one freeze-thaw cycle (freezing in liquid N_2 followed by thawing at 37°C). Nuclei were separated by centrifugation. The supernatant was adjusted to 70 mM $MgCl_2$, and the cleavage reaction was carried out after the addition of ribozymes. The 70 mM $MgCl_2$ effectively inhibits RNase activity in the cytoplasmic RNA-protein preparation and allows for the same efficiency of cleavage as 10 mM $MgCl_2$ (13). This cytoplasmic RNA preparation should maintain physiological folding and protein packaging of mRNAs.

Cleavage was carried out at physiological pH (50 mM Tris-HCl [pH 7.5]) at 37°C for 1 h in a 15-μl reaction volume with or without prior heat denaturation (for 90 s at 95°C). The cleavage products were analyzed in a 2% ethidium bromide-stained NuSieve agarose gel and could be detected as a smear between the 18S and 28S rRNAs and below the 18S rRNA. In some cases the 5'-OH groups of cleavage products were phosporylated with [^{32}P]ATP by using polynucleotide kinase and quantified on a Fuji Phosphorimager after polyacrylamide gel electrophoresis.

Four different cleavage reactions were performed. The first involved total RNA plus ribozymes transcribed by T7 polymerase. Purified total RNA (1 μg per reaction) and 10 μg of vaL (as control) or vaLRz (library) RNA, which were synthesized by T7 polymerase, were mixed and incubated in 50 mM Tris-HCl–10 mM $MgCl_2$ for 1 h at 37°C with or without prior heat denaturation. No significant self-digestion of ribozymes was observed. The ribozyme cleavage was three times more efficient when the reaction mixture was heat denatured. Without addition of 10 mM $MgCl_2$, no specific cleavage reaction was detected. For further anal-

yses, cleavage products were used without prior heat denaturation. The second cleavage reaction involved cytoplasmic RNA plus ribozymes transcribed by T7 polymerase. The cytoplasmic RNA-protein fraction of 10^5 cells was incubated with 10 μg of vaL (as control) or vaLRz (library) RNA, which were synthesized by T7 polymerase for the cleavage reaction without heat denaturation. One-fifth the amount of cleavage products from the first reaction was observed, probably because of a lower yield of RNA. The third and fourth cleavage reactions involved total RNA or cytoplasmic RNA, respectively, plus ribozymes transcribed by pol III. Purified total RNA (1 μg per reaction) or the cytoplasmic RNA-protein fraction of 10^5 cells was incubated with 1 μg of vaL (as control) or vaLRz (library) RNA, which were synthesized by pol III for the cleavage reaction without heat denaturation.

After the reaction, RNA was purified with oligo(dT)-cellulose (PolyATract mRNA Isolation System; Promega) as specified in the technical manual. After purification, 0.05 to 0.5 μg of RNA was annealed with 2.5 μM oligo(dT) primer (Promega) for 10 min at 70°C, and unbound primer was separated by centrifugation through a 30,000-molecular-weight-cutoff filter unit (Millipore). Reverse transcription was performed with 200 U of superscript II reverse transcriptase (Bethesda Research Laboratories) at 37°C for 1 h. To eliminate free primers, cDNA-RNA hybrids were purified with Gene Clean (Bio 101). The cDNA-RNA hybrid in 30 μl of H_2O was boiled for 2 min and cooled on ice. Tailing was carried out for 15 min at 37°C in a total volume of 50 μl containing 200 μM dGTP, 20 U of terminal deoxynucleotidyltransferase (Bethesda Research Laboratories), and tailing buffer.

A 5% portion of the tailing-reaction mixture was used for the first PCR with 200 μM dNTP, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase in buffer containing 2% dimethyl sulfoxide, 50 mM KCl, 10 mM Tris-HCl (pH 7.9), and 0.1% Triton X-100 in a 100-μl reaction volume. The initial seven cycles (30 s at 95°C, 30 s at 42°C, and 90 s at 72°C) were run in the presence of 15 μM C-Primer (5'GAGAATTCTAGAGGATCCCCCCCCCCCC3') only. After addition of 250 μM hGH-specific primer (5'GAGAATTCCAAGGCCAGGAGAGGCA CTGGGGA3'), which is specific for a region immediately upstream of the poly(A) signal of the genomic hGH gene, PCR was done for 40 cycles (60 s at 94°C and 90 s at 72°C, or 60 s at 95°C, 45 s at 65°C, and 60 s at 72°C). The reaction mixture was run on a native 5% PAA gel in Tris-acetate buffer, and the specific bands were cut out and purified with Gene Clean. The resulting fragments were cloned in the pGEM-T II vector system (Promega). Clones corresponding to the 5' end of individual RNA downstream cleavage products were sequenced with the fmole sequencing kit (Promega).

Amplification of ribozymes. Ribozyme genes from the library were amplified as follows. PCR was performed by incubating 50 ng of plasmids from the ribozyme library, 20 μM each upstream and downstream primer (which are specific for the sequences around the GTC.CTC site [see Fig. 9]), and 2.5 U of *Taq* polymerase in buffer containing 2% dimethyl sulfoxide, 50 mM KCl, 10 mM Tris-HCl (pH 7.9), and 0.1% Triton X-100 in a 100-μl reaction volume for 40 cycles (45 s at 95°C, 45 s at 52°C, and 45 s at 72°C). Specific fragments were prepared as described above, digested with *XhoI-NsiI*, cloned into the *SalI* and *PstI* sites of GvaL, and sequenced with the T7 promoter primer.

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium containing 200 mM asparagine, 200 mM proline, 200 mM glutamine, and 10% fetal calf serum (GIBCO, Grand Island, N.Y.) under a 5% CO_2 atmosphere.

Transfection. Plasmids were purified by two rounds of cesium chloride gradient centrifugation. For transfection of CHO cells, a modification of the standard calcium phosphate coprecipitation method was used (28). DNA (10 μg) in 220 μl of H_2O was mixed with 30 μl of 2 M $CaCl_2$, and 250 μl of 2× HBS (50 mM HEPES, 280 mM NaCl, 1.5 mM sodium phosphate [equal amounts of mono- and dibasic] [pH 6.96]) was added dropwise while the mixture was vortexed. The precipitate was added to 5 ml of culture medium in 25-cm^2 tissue culture flasks containing 2.5 × 10^5 cells that had been seeded the day before. Cells were transfected with a mixture of 8 μg of test plasmid, 1 μg of pCMVluc (31) as the transfection control, and 1 μg of pSV2neo (39). After 48 h, 1/10 of the cells were subjected to selection with 600 μg of G418 per ml to generate pools of about 100 colonies in order to test for stable expression levels. hGH levels were estimated by enzyme-linked immunosorbent assay (ELISA) as described previously (29).

RESULTS

Construction and function of the expression cassette. The expression unit for the ribozyme library should guarantee high-level expression and stability of the expressed RNA (ribozyme) molecules. For high-level expression, we decided to use alternatively either transcription by pol III or our previously developed T7 promoter-polymerase system for mammalian cells (28–30). As a gene for embedding the antisense-ribozyme sequences, we chose the human adenovirus type 2-associated vaRNA I gene. The 155-nt vaRNA I forms a stable secondary structure and is associated with proteins forming small nuclear ribonucleoprotein (25). These features are expected to cause high levels of stability and resistance against degradation nucleases.

In lytic viral infections, vaRNA accumulates at high concentrations in the cytoplasm. vaRNA is synthesized by RNA pol III from a promoter within the transcribed sequence with characteristic and essential elements for transcriptional initiation the regions at positions +10 to +16 and +58 to +68 (Fig. boxes A and B). The promoter is active in all cell types (2 Transcription by RNA pol III terminates at an oligo(dT) tra Our expectation was that ribozymes as part of vaRNA wou be expressed in a stable form and would also be transported the cytoplasm in a manner similar to that for spliced mRN To this end, we cloned the genes for vaRNA I and II into t vector pGem7zf− behind the T7 promoter, allowing for bo in vitro and in vivo transcription by T7 RNA polymerase addition to pol III transcription.

Since others have shown that intramolecular base pairi can destroy the ribozyme structure (44), a stable stem-lo structure-forming sequence was inserted in the center of t expressed part of the RNA gene behind the pol III promo (Fig. 1). The ribozyme gene was positioned within the loop allow the structure of the ribozyme to form independently the structure of flanking regions. As the test ribozyme, designed one against a region within exon IV of the genon hGH gene (see Fig. 3A). The regions of complementar flanking the GUC site were 14 and 13 nt long.

The construct was tested for correct pol III-dependent tra scription of the modified vaRNA genes in a HeLa cell extra As shown in Fig. 2, in vitro transcription terminates exactly the oligo(dT) whether the template was linear or circul (lanes 1 to 4). Inhibition of in vitro transcription by only hi concentrations of α-amanitin (250 μg/ml) indicates that t RNA was indeed transcribed by pol III (lanes 7 to 9). Mo fication of the vaRNA gene by the loop oligonucleotide a the ribozyme did not affect transcription by pol III (lanes and 11).

Function and stability of the chimeric ribozyme RNA. V next tested for the function of this empirically designed hGl specific ribozyme (Fig. 3A) in vitro. To study the effects flanking sequences, we used different constructs (Fig. 3B) f transcription by either T7 polymerase or pol III. Efficie cleavage of hGH-specific RNA (Fig. 3C, lane 1) was observe with the transcribed ribozyme sequence only (lane 2) but n with an attached *neo* gene or with the flanking vaRNA s quences (lanes 3 to 5). In contrast, the ribozyme inserted in the GvaL vector containing the loop region is able to fold ar bind to the substrate, leading to efficient cleavage (lanes 6 ar 7), even when transcription is from a circular template (lane 7 This indicates that an active ribozyme structure and most proi ably the stabilizing structures of the ends of vaRNA are co rectly generated by T7 polymerase transcription with our e pression cassette. Thus, the T7 expression system should als be useful for in vivo expression of ribozymes.

In vivo expression of the hGH-specific ribozyme by pol III T7 polymerase was studied in transient-transfection assays a well as in assays with stable clones. We detected efficient tran port of the ribozyme transcribed by pol III to the cytoplas (Fig. 4A), reasonable stability of the ribozyme (Fig. 4B), an high-level stable expression in the mammalian T7 expressio system (Fig. 4C). In the last expression system, we estimated steady-state level of 10^5 ribozyme molecules per cell. The hig level of stable expression does not affect the viability of th cells, as indicated by cell-doubling rates and expression of luciferase control gene (data not shown).

To analyze the function of the ribozyme vaLRz in vivo, w cotransfected GvaLRz, pCMVhGH (29), and pSV2neo at

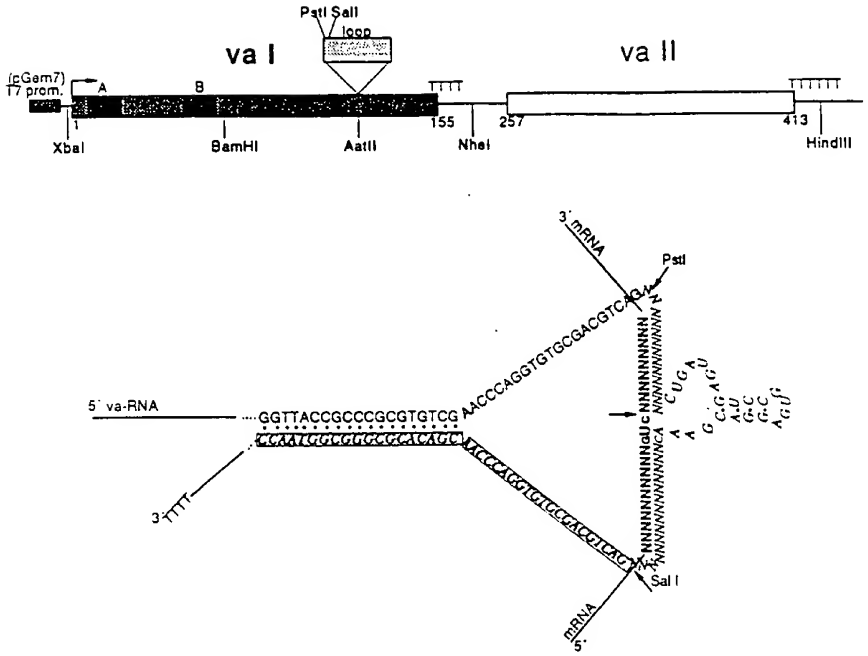

FIG. 1. Design of the expression cassette for ribozymes. The genes for vaI and vaII were cloned behind the T7 promoter in pGEM-7. pol III transcription starts from an intragenic promoter containing the characteristic A and B elements and stops at an oligo(dT) tract at the 3′ end. An oligonucleotide coding for a "loop" was cloned into the *Aat*II site. The RNA region corresponding to this sequence would form a stable stem structure with an upstream region of the nascent vaRNA. The ribozyme gene was inserted between the *Pst*I and *Sal*I sites within the loop, thereby allowing for correct folding of the ribozyme and its flanking sequences independent of the structure of the vaRNA.

ratio of 10:1:1 into CHO cells. However, we could not find a significant decrease in the amount of hGH protein and messenger as a result of ribozyme cleavage in either stable or transient expression (data not shown). This led us to conclude that the target sequence within the hGH RNA is probably not accessible for hybridization with the ribozyme at the selected site. This conclusion is also supported by the in vitro assay, in which cleavage was possible only after heat denaturation of the RNA.

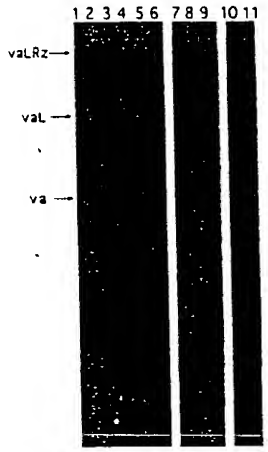

FIG. 2. RNA pol III-dependent in vitro transcription of vaRNA. Plasmid DNA (2 μg) was transcribed with 5 μl of HeLa extract (15 mg of protein per ml) in the presence of 10 μCi of [^{32}P]CTP. Products were separated on a 6% denaturing PAA gel. The band on the bottom is HeLa cell specific. va, vaRNA; vaL, vaRNA plus loop; vaLRz, vaRNA plus loop plus ribozyme. Lanes: 1, Gva linearized with *Nhe*I (Fig. 1); 2, Gva linearized with *Hin*dIII; 3 and 4, circular Gva plasmid (different plasmid preparations); 5 and 6, circular GvaL plasmid; 7, GvaL without α-amanitin; 8, GvaL plus 1 μg of α-amanitin per ml; 9, GvaL plus 250 μg of α-amanitin per ml; 10 and 11, circular GvaLRz plasmid (different plasmid preparations).

Creation of a functional ribozyme library. We first generated a library of ribozyme genes with random sequences in the flanking region (Fig. 5). Ribozyme genes were synthesized as single-stranded oligonucleotides overlapping within the central part of the catalytic region. After annealing, full-length double-stranded oligonucleotides were generated by a fill-in reaction with *Taq* polymerase. In all positions of the flanking regions, all four nucleotides were incorporated with the same probability, with the exception of a CA dinucleotide at the proximal end of the left flanking region determining part of the cleavage site (GUC). Since the recognition site of hammerhead ribozymes can be NUH (20, 33), we incorporated a mixture of all nucleotides in the N position. The maximal complementarity within the flanking regions is 26 nt (13 and 13). After cloning of the double-stranded ribozyme oligonucleotides into the GvaL cassette, 10^9 plasmid clones with different sequences were generated. The quality of the ribozyme plasmid library was checked by sequencing 50 individual clones (data not shown). The size of the library should allow us to find ribozyme variants for hGH RNA with a region of complementarity to a target of 15 bp (8 and 7 bp) around the NUH site for all 156 potential NUH sites within the hGH target RNA (1,600 nt). For ribozymes from the library with 20-bp (10 and 10 bp) complementarity around the NUH, the probability of finding at least one cleavage site within hGH RNA would be 13%.

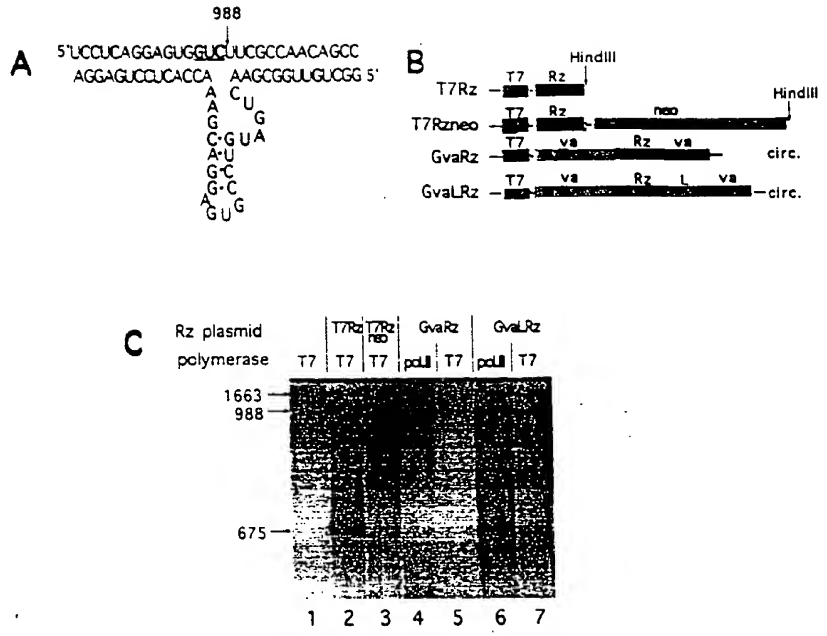

FIG. 3. Cleavage of hGH RNA by a specific ribozyme in vitro. (A) Structure of the ribozyme specific for a 27-nt region around the GUC at position 988 within exon IV of hGH RNA (38). (B) Maps of plasmid templates for ribozyme synthesis by in vitro transcription with pol III (HeLa extract) and T7 RNA polymerase. Plasmids T7Rz and T7Rzneo were linearized by *Hin*dIII digestion. GvaRz and GvaLRz were used in a circular form. va, val; L, loop. (C) hGH RNA was synthesized from a linear (*Sst*I-cut) genomic hGH gene (1.663 nt) by in vitro transcription with T7 RNA polymerase (with 0.2 μCi of [^{32}P]CTP per μg of RNA). An equimolar mixture (100 nM) of substrate and ribozyme was incubated at 37°C in 50 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$ for 30 min with prior heat denaturation (90 s at 95°C). After cleavage, the RNAs were purified by phenol extraction and ethanol precipitation and separated on a 6% denaturing PAA gel. hGH target RNA and ribozyme cleavage products (988 and 675 nt) are indicated.

The total ribozyme plasmid library was transcribed in vitro by T7 polymerase or pol III, and transcripts were purified. The ribozymes were synthesized at a quantity allowing us to adjust the concentration of each ribozyme variant in the mixture to 50 to 100 pM during the cleavage reaction with cellular RNA. This ribozyme concentration would be sufficient to generate an amount of cleavage product from a moderately expressed mRNA which is detectable by amplification and cloning of 5′ ends (see Materials and Methods).

To test for the overall ribozyme activity of our library, we incubated either total-cell RNA or a cytoplasmic RNA-protein fraction with the in vitro transcribed ribozymes (Fig. 6). The results can be summarized as follows. Efficient cleavage of total RNA was dependent on prior heat denaturation of the target RNA and on the presence of magnesium ions (Fig. 6A). Massive degradation was observed in the cytoplasmic RNA-protein preparation, which could be inhibited by high magnesium concentrations (70 mM). However, relatively little cleavage by ribozymes was observed under these conditions with either T7 polymerase transcripts (Fig. 6B) or pol III transcripts (Fig. 6C) compared with cleavage of purified total RNA.

RNAs from various incubations (Fig. 6A, lanes 8 and 9; Fig. 6B, lanes 4 and 5; Fig. 6C, lanes 2 and 3) were analyzed by the rapid amplification of cDNA ends (RACE) technique (3, 24) as follows. The 3′ mRNA fragments were purified by oligo(dT) chromatography and reverse transcribed with oligo(dT) as a primer. Following tailing with dG and annealing of an oligo(C)-linker primer, double-stranded cDNA was generated and was further amplified in the presence of a gene-specific downstream primer (Fig. 5). As a first example, we have chosen to identify hGH-specific cleavage products by using RNA

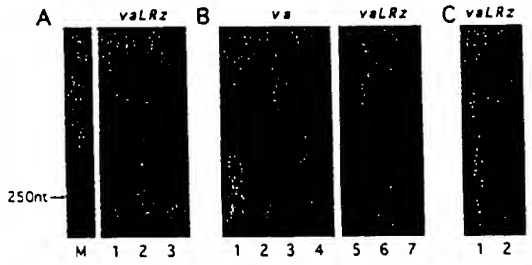

FIG. 4. Northern blot analysis of vaRNA expressed in mouse hepatocytes. Gva or GvaLRz plasmids were transfected into HepSV40 cells (32) together with the selection marker pSV2neo (39) (ratio 50:1) and pCMVluc (31) as the transfection standard and harvested for RNA preparation at day 3 or 10 after transfection. An aliquot of cells was selected for stable expression with 1 mg of G418 per ml over 4 weeks. For RNA preparation, a pool of more than 100 stable clones per transfection was used. A 5-μg portion of RNA was loaded per lane on a 1.5%-denaturing agarose gel, and filter hybridization was carried out with a va gene-specific DNA probe. vaRNA is 155 nt; vaLRz RNA is 250 nt. The two bands in the upper half of all lanes are 18S and 28S rRNA, which cross-hybridize with the va gene probe. (A) Analysis of nuclear (lane 3) and cytoplasmic (lane 2) RNA fractions and control cell RNA (lane 1). Lane M contains 20 pg of marker RNA (in vitro T7 polymerase transcript from Gva digested with *Nhe*I) (250 nt). Cells were harvested for RNA preparation at day 3. (B) Comparison of stabilities of vaRNA and vaLRz RNA. Lanes: 1 and 5, vaLRz RNA (cells harvested at day 3); 2 and 6, vaLRz RNA (cells harvested at day 10); 3 and 7, vaLRz RNA (cells harvested from more than 100 stable clones); 4, control HepSV40 cells. (C) Stable expression of GvaLRz with the T7 polymerase expression system. HepSV40 cells were cotransfected with GvaLRz pMTTN-pT7neo pGpolioluc (10:10:1:1) and selected with G418 for 4 weeks. pMTTN (28) and pGpolioluc (3) were as described previously. Lanes 1 and 2 contain material from different transfections. Quantitative analysis of bands was done with a Fuji Phosphorimager.

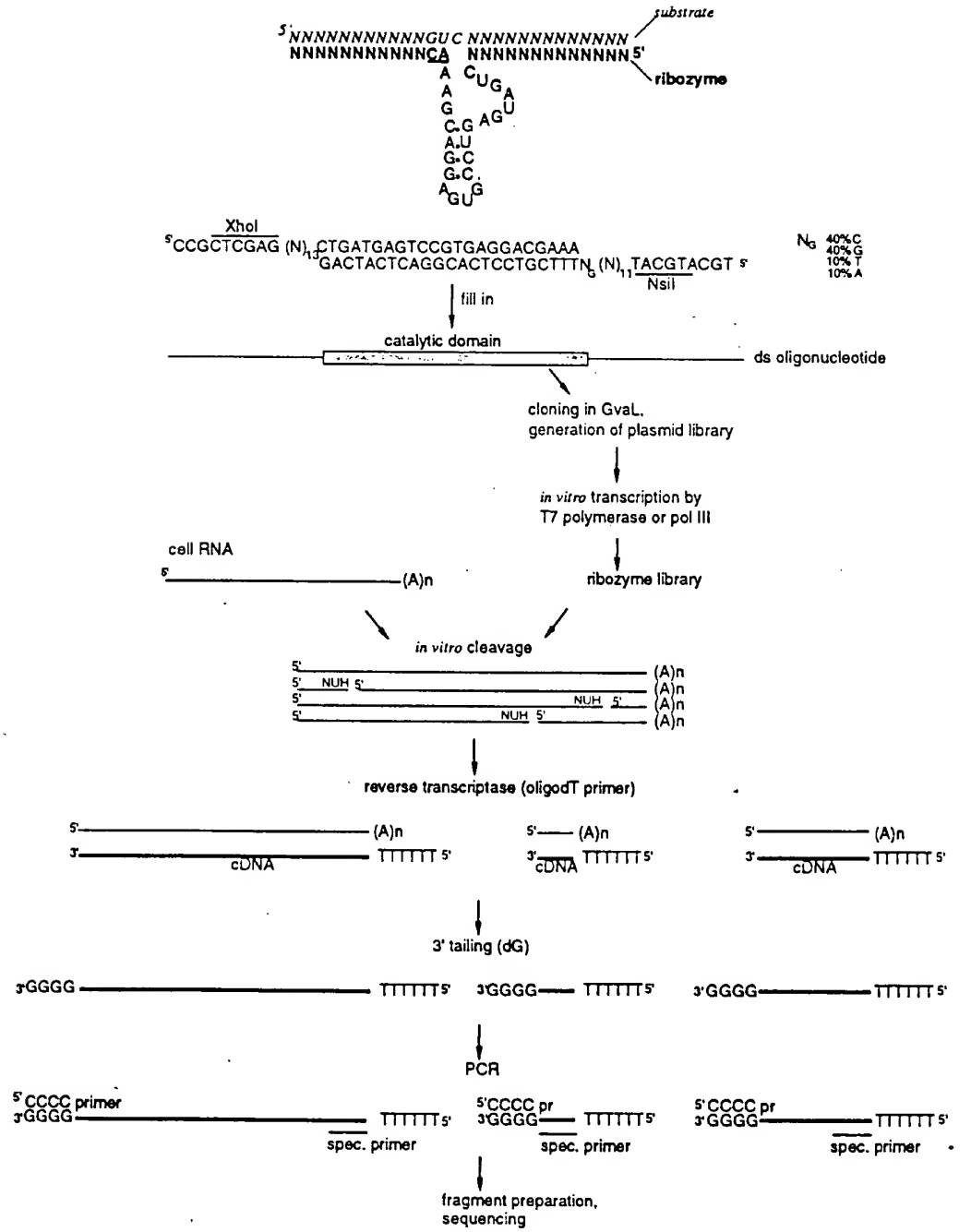

FIG. 5. Strategy for isolation of target-selected ribozymes. The upper part of the figure shows the structure of the designed ribozyme pool; below are shown the actual sequences of the two oligonucleotides which were annealed and extended to generate the double-stranded (ds) ribozyme genes. The resulting fragment mixture was cloned into the GvaL cassette as *XhoI-NsiI* fragments. A library of 10^9 different variants was generated. The ribozymes were synthesized in vitro by T7 polymerase or pol III from a HeLa extract. RNA from CHO cells which stably express the hGH gene (5,000 RNA copies per cell) was used as the target. Purified RNA was incubated with the in vitro synthesized ribozyme library. After the purification of cleavage products on an oligo(dT) column, the 5' ends of downstream cleavage products were analyzed by the RACE technique (3, 24) as follows. After the reverse transcription reaction with oligo(dT) primers, the cDNAs were tailed at the 3' end with dG, amplified with an oligo(dC) and nested hGH-specific (spec.) primers, cloned into pGEM-T (Promega), and sequenced. The sequences should start immediately at the NUH recognition sites within the hGH RNA. The gene coding for the ribozyme which had mediated the cleavage at a selected site was amplified by PCR from the ribozyme plasmid library with degenerated primers specific to the flanking region of ribozyme genes (see Fig. 9).

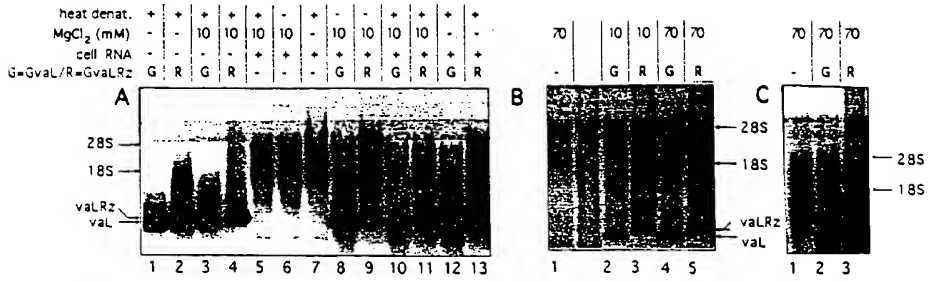

FIG. 6. Detection of ribozyme activity of the library in vitro. Cellular RNA was cleaved at physiological pH (50 mM Tris-HCl [pH 7.5]) at 37°C for 1 h with or without prior heat denaturation (90 s at 95°C) in a 15-μl reaction volume. Samples were run on a 2% NuSieve agarose gel stained with ethidium bromide. Cleavage products are detectable as a smear between the 28S and 18S and below the 18S rRNA bands. (A) Purified total RNA (1 μg per reaction) was used as the target. Ribozyme GvaLRz (R) was used as a T7 transcript (20 μg per reaction). The in vitro transcript of GvaL (G) served as a control. (B) The cytoplasmic RNA-protein fraction was used as the target. A total of 10^5 cells were lysed in 50 mM Tris-HCl (pH 7.5) for 10 min on ice, frozen in liquid nitrogen, and thawed at 37°C; the nuclei were removed by centrifugation. As the ribozyme, a T7 transcript of GvaLRz (10 μg) was used. For effective inhibition of RNase activity in the cytoplasmic RNA-protein preparation, 70 mM $MgCl_2$ was added to the cleavage reaction mixture. This Mg^{2+} concentration allows for the same efficiency of cleavage as 10 mM $MgCl_2$ and should not influence the RNA structure considerably (11). It is not known whether folding of reaction partners in the presence of 70 mM $MgCl_2$ is close to that under physiological conditions. (C) Cytoplasmic RNA. Ribozyme was generated by pol III transcription (2 μg per sample).

from an overproducing clone (29). The results of amplification of hGH-specific fragments after incubation of either cytoplasmic or total RNA with different ribozyme transcripts are given in Fig. 7. Six bands of the expected size corresponding to predicted fragments of hGH nuclear RNA or mRNA were detectable in incubations with total RNA. Two of the fragments seem to represent intron-containing sequences (I1 and I2), whereas the others are most probably derived from exons (E1 to E4). In incubations with cytoplasmic RNA, a strong E1 band and a weak E2 band were observed. The absence of bands E3 and E4 could be due to inaccessibility of the respective cleavage sites in the mRNA structure. Fragments E1 to E5 (E5 was derived from another size fractionation) and I1 and I2 were cut out from the gel and cloned into pGEM-T, and 20 individual clones were sequenced from every fragment. About 50% of the clones represented specific cleavage products with

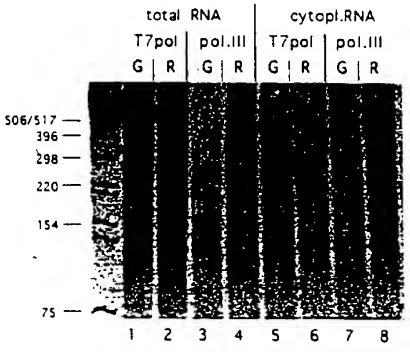

FIG. 7. Detection of hGH-specific RNA fragments after cleavage of cellular RNA in vitro by ribozymes from the library. Total or cytoplasmic RNA preparations from hGH-producing cells were incubated with ribozymes from the library, which were transcribed by either pol III or T7 polymerase as in Fig. 6 (see Materials and Methods). hGH-specific 3′ fragments were reverse transcribed and amplified by PCR as outlined in the legend to Fig. 5. Conditions for PCR were chosen to produce fragments mainly of less than 1,000 bp. PCR products were separated on a 6% PAA gel (lanes 1 to 8); fragment size markers were included in the leftmost lane. G, GvaL as the template; R, GvaLRz library as the template. Designation of bands E1 to E4 corresponds to specific products derived from cleavage in exon sequences, and I1 and I2 correspond to cleavage within intron sequences as confirmed by sequencing. The band at approximately 400 bp in lanes 5 to 8 represents a nonspecific amplification product. Note that T7 polymerase transcripts are easier to detect and that more cleavage sites are accessible in total RNA than in cytoplasmic RNA.

the cleavage site at a CUC (five fragments) or a GUC (two fragments) site. The locations of the cleavage sites are depicted in Fig. 8. Among the 156 NUH sites within the hGH mRNA are 72 CUC and 29 GUC sites (2.5:1 ratio). The same ratio was also found for the fragments analyzed, suggesting that both sites are used by hammerhead ribozymes with similar efficiency. Interestingly, the secondary structure of the hGH RNA fragment predicted by the computer suggests that all experimentally detected cleavage sites are located in loops or other single-stranded regions (Fig. 8).

Identification of specific ribozymes in the library. The method for isolating ribozymes is based on the assumption that the sequence of the best ribozyme is complementary (Watson-Crick) to the respective cleavage site. To identify the ribozymes in the library which have produced a particular fragment, we have focused on those able to generate fragment E1 from hGH mRNA. To this end, we have designed two primers for both sides of the cleavage site with 8- and 7-nt complementarity for the target sequence (Fig. 9). Using these two oligonucleotides, we have amplified ribozyme sequences from the library, cut them with *Nsi*I-*Xho*I, cloned them between the *Pst*I and *Sal*I sites of the pGvaL vector, and sequenced 50 clones. Three different ribozyme clones were detected (Fig. 9), indicating that the library is representative and contains different ribozymes for the one target site selected. A similar result was obtained for ribozymes specific for fragments E5 and I1 (data not shown). For further studies on the function of the ribozymes, we generated a mutant of ribozyme E1(8/7) with a mutation in the hammerhead region (A → G) (Fig. 9) which is known to abolish cleavage activity completely in vitro (34) and in vivo (40).

Individual ribozymes were tested for target-specific cleavage activity in vitro with equimolar amounts of in vitro transcribed target RNA and ribozyme. The three ribozymes specific for different target sites were all active in vitro without heat denaturation (Fig. 10A). All three different variants of the E1-specific ribozyme generated the two predicted cleavage products of 1,017 and 646 nt. No obvious difference in the efficiency of cleavage in vitro as a function of the length of the homology region was observed with the E1 ribozyme (Fig. 10B). Obviously, only single-turnover reactions are detectable under the conditions used for in vitro cleavage.

Function of target-selected ribozymes in vivo. To investigate the in vivo function of the selected ribozymes, all GvaLRz

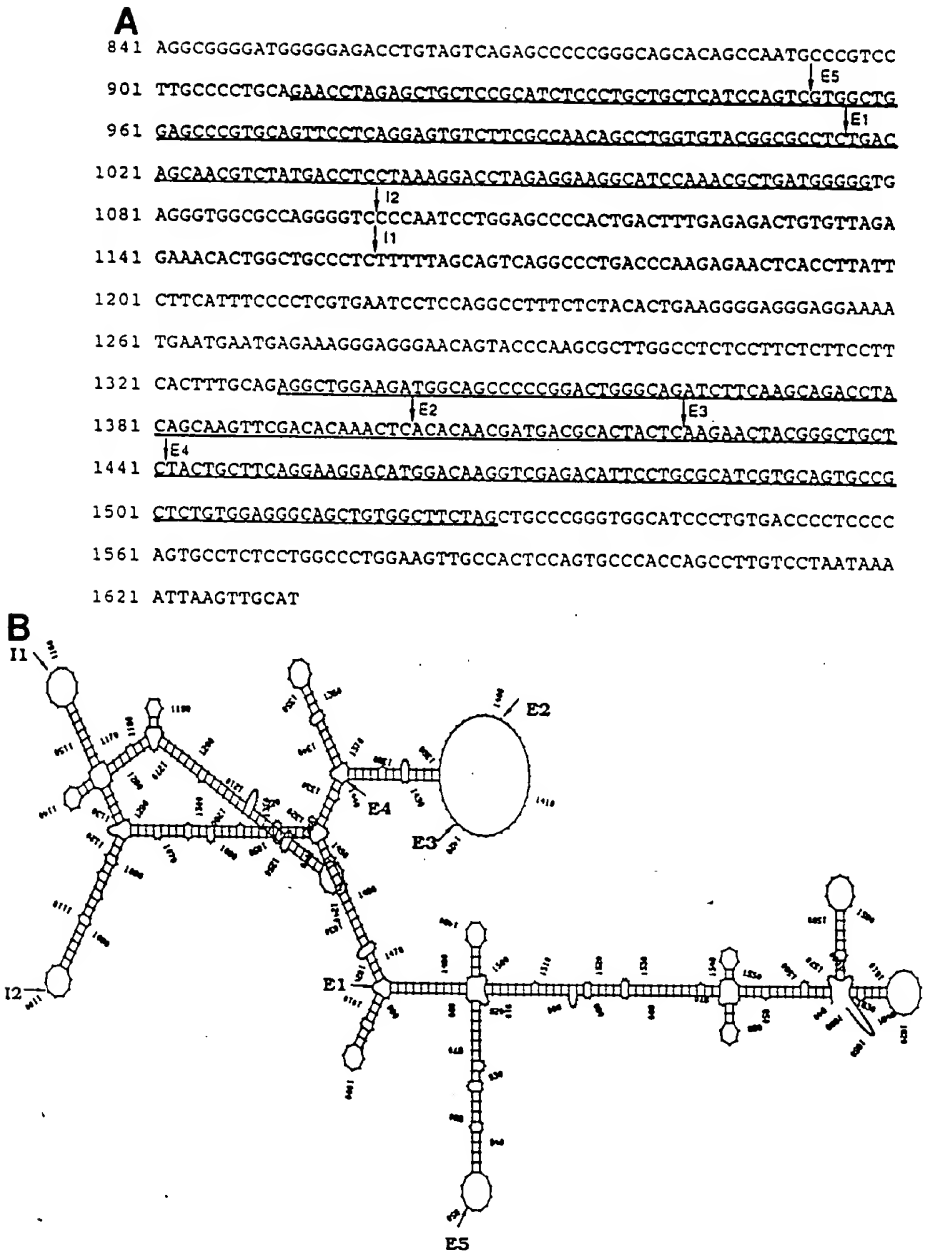

FIG. 8. Localization of ribozyme cleavage sites within the hGH mRNA. Fragments E1 to E4, I1, and I2 (Fig. 7, lane 2) were cut out from the gel, purified, and cloned in pGEM-T. Twenty clones were sequenced from each fragment. The sequences of about 50% of the clones started at either a CUC or a GUC. The other 50% probably represent degradation products. (A) The cleavage sites corresponding to the fragments are indicated in the partial sequence of hGH. E1, CTC 1017 (exon IV); E2, CTC 1401 (exon V); E3, CTC 1422 (exon V); E4, CTC 1441 (exon V); E5 (derived from a separate experiment), GTC 952 (exon IV); I2, CTC 1099 (intron IV); I1, GTC 1159 (intron IV). (B) Model of the secondary structure of hGH RNA generated by the program MFOLD (HUSAR). The graphic representation was obtained by PLOTFOLD (26).

plasmids were transfected into CHO cells together with pCMVhGH and pSV2neo (50:50:1) and pCMVluc as the transfection control. Expression of ribozymes would be driven by the pol III promoter. For T7-dependent expression (29), we transfected a mixture of pCMVhGH/GvaLRz/pMTT7N/pT7neo and pGpolioluc (1:10:10:1:1). pT7neo provides T7-dependent selection, and pGpolioluc codes for T7-dependent luciferase activity. Cell culture media were assayed for hGH-specific protein by ELISA at day 3 after transfection. An aliquot of cells was selected for stable expression with 600 μg of G418 per ml over 4 weeks. Supernatants of a pool of more than 100 stable clones were used in each case for determination of hGH levels. The results are summarized in Table 1.

All ribozymes reduced the level of hGH production significantly. However, interesting and important differences were detected. For the three different E1-specific ribozymes the

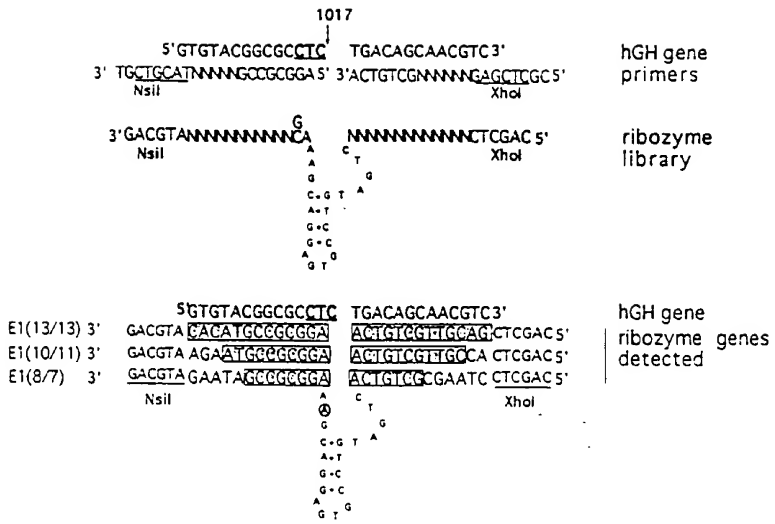

FIG. 9. Isolation of ribozyme genes from the library. The figure shows the target sequence and designed primers for ribozyme E1; below this are the sequences of the three different ribozymes isolated from the library. Ribozyme sequences were amplified from the library, cut with *Xho*I-*Nsi*I, and cloned into the *Sal*I and *Pst*I sites of GvaL, and the clones were sequenced. Among the 50 clones sequenced, only three different types of ribozymes with homologies to the target sequence of 7 or 8 to 13 nt were found

strongest effect was observed for the one with the shortest homology region (8 and 7 bp) in all four settings. Although the effect was more pronounced in transient-expression assays (7%) than in stable-expression assays (25%) for the pol III-based expression, the opposite was observed for T7-dependent expression. Here a very strong inhibition of hGH production was detected in stable clones which were selected for T7 polymerase expression. For pol III transcription, the amount of ribozymes was about 100-fold greater in the transient-expression assay (Fig. 4B) than in the stable-expression system. The reduction of hGH production in stable clones was more than 100-fold stronger with the T7-polymerase expression system. In this system, selection for stable T7 polymerase expression was required to guarantee high-level expression of ribozyme genes from the T7 promoter. The mutant ribozyme had a marginal effect, which could be ascribed to an antisense effect. The ribozyme directed against an intron sequence (I1) had only minimal effect. The ribozymes directed against hGH mRNA are probably highly specific. The expression of luciferase, whose mRNA also has potential cleavage sites, was not affected (data not shown).

Finally, we analyzed the in vivo effect of the ribozyme E1(8/7) on the RNA level by Northern blotting (Fig. 11). More than 50% reduction of hGH-specific RNA was detected for pol III-dependent ribozyme expression (lane 4). A much stronger reduction of the level of full-length RNA was detected in individual clones for T7-dependent expression of the ribozyme (lanes 6 to 10). However, no specific cleavage products could be identified in this assay, probably because of instability of RNA fragments with a 2',3' cyclic phosphate and a 5'-OH group (40).

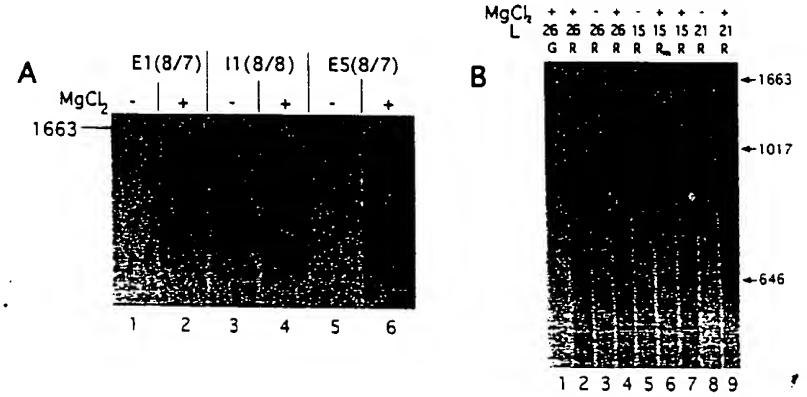

FIG. 10. Cleavage in vitro of hGH-specific RNA by ribozymes isolated from the library. (A) Cleavage with three different selected ribozymes (E1, I1, and E5). Ribozymes were transcribed by T7 polymerase from a selected clone and incubated with in vitro-transcribed hGH RNA at the same molarity (100 nM each) for 20 min at 37°C without heat denaturation. Samples were run on a denaturing 6% PAA gel. The resulting fragments have the expected sizes (E1, 1,017 and 646 nt; I1, 1,095 and 564 nt; E5, 952 and 711 nt). (B) Cleavage of hGH RNA by E1 ribozymes with different lengths of complementary regions. Incubation was as for panel A. Fragment separation was done on a 4% denaturing PAA gel. L, length of complementary regions of E1 ribozyme: 26, 13/13; 15, 8/7; 21, 10/11; G, GvaL; R, E1 ribozyme gene in GvaL as a template; Rm, E1(8/7)mut. Note that the two specific cleavage products are detectable only after incubation with ribozymes in the presence of magnesium

TABLE 1. Effect of ribozyme expression in vivo on the level of hGH secretion[a]

Expression system[b]	hGH secretion (%)[c] with:			
	pol III		T7 polymerase	
	Transient	Stable	Transient	Stable
GvaL	100	100	100	100
E1(13/13)	36	85	78	2
E1(10/11)	12	50	NT	NT
E1(8/7)	7	25	75	0.2
E1(8/7)mut	95	92	90	87
E1(8/8)	42	78	NT	NT
E5(8/7)	32	50	NT	NT

[a] Transfections of CHO cells with pCMVhGH, ribozyme or control construct, and pT7neo were carried out as described in Materials and Methods. Transient expression was tested after 3 days, and stable expression was tested after selection with G418 for 4 weeks.

[b] Numbers in parentheses refer to numbers of nucleotides on both sides of the hammerhead.

[c] hGH levels were determined by ELISA (limit of detection, 3 ng/ml). The levels of hGH obtained with pCMVhGH + GvaL were taken as 100% (transient, 7 μg of hGH per ml per h; 24 stable, 2 μg of hGH per ml per 24 h). The presence of almost equal copy numbers of the target gene and the ribozyme gene in stable clones was confirmed by Southern blotting (results not shown). NT, not tested.

DISCUSSION

Strategy for expression of target-selected ribozymes. We have developed a new strategy for generating ribozymes which are highly specific and active in vivo. This strategy is based on two major achievements. First, we have constructed an optimized expression cassette for ribozymes in which the ribozyme sequence is embedded in a stable loop region which, in turn, is part of vaRNA gene. In addition, we can increase the level of expression of the ribozyme by driving its expression from a T7 promoter, using cotransfection with a T7 polymerase gene. The T7 expression system guarantees very high levels of ribozyme expression. The second part of the strategy involves the application of a library of ribozymes flanked by random sequences which are cloned into the loop region of the expression cassette. The unique feature of the strategy is the selection of an accessible cleavage site in the target RNA by a ribozyme from a library which contains 10^9 individual ribozymes with random sequences in the target-binding arms. With this strategy, it is also possible to isolate an optimal ribozyme for the accessible cleavage site. At least one of the isolated ribozymes should have the optimal structure for allowing efficient cleavage of the target RNA.

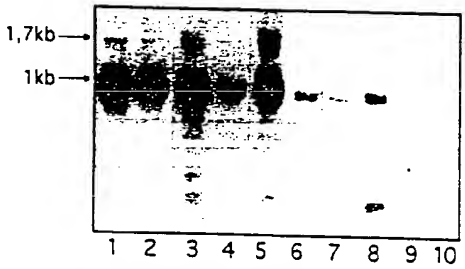

FIG. 11. Detection of hGH-specific mRNA after stable expression of the E1 ribozyme. Cells were cotransfected with pCMVhGH, ribozyme or control construct, pMTTTN, and pT7neo and selected with G418; then RNA was isolated from pooled colonies. A 5-μg portion of total RNA was run in each lane. After electrophoresis the gel was blotted to Hybond N filters and hybridized with an hGH-specific DNA probe labelled with [^{32}P]ATP by random priming. Both the unspliced nuclear hGH RNA (1.7 kb) and the spliced mRNA (1.0 kb) are visible. The latter band in control cells corresponds to about 5,000 copies per cell. Lanes: 1, GvaL; 2, E1(8/7)mut (stable pol III expression); 3, GvaL (stable pol III expression); 4, E1(8/7) (stable pol III expression); 5, GvaL (stable T7 expression); 6 to 10, different clones with E1(8/7) (stable T7 expression). The clones shown in lanes 6 to 10 have comparable amounts of the hGH gene integrated as demonstrated by Southern blotting (results not shown).

Our initial experiment in which we used a predesigned ribozyme for a selected target site within the mRNA of hGH worked perfectly in vitro but failed completely in vivo. This prompted us to design the described strategy for selection of target sites and isolation of corresponding ribozymes from a library. By applying this strategy, we should be able to determine two things: first, the regions of a certain target RNA that are accessible for cleavage by a ribozyme in vivo, and second, the optimal length of the complementary flanking regions of the hammerhead allowing for efficient cleavage and dissociation from the target. The strategy also allows us to find simultaneously several ribozymes directed against different target sites in a particular mRNA. This strategy can be adapted to any other target RNA and requires only the use of gene-specific primers to isolate the respective ribozymes.

All ribozymes specific for hGH selected by this method were able to cleave the hGH RNA in vitro without prior denaturation, and they generated fragments of expected sizes. The ability of the selected ribozymes to cleave without the need for denaturation indicates that active ribozymes in the correct conformation are probably also generated in the intracellular milieu.

Expression cassette. On the basis of the experience of others, we have constructed an expression cassette with a suitable structure for high-level expression of exposed ribozymes. High copy numbers of expressed ribozymes were previously achieved by expression based on pol II-dependent transcription (7), pol III-dependent transcription (12), and transcription by a cytoplasmic RNA polymerase of T7 phage (27). The pol III-dependent transcription of a ribozyme as part of U1-RNA was recently shown to be highly effective (5). Here we have used identical constructs to compare the efficiency of pol III-dependent transcription and transcription by a nuclear T7 RNA polymerase. We can confirm the biological function of ribozymes transcribed by pol III. However, even higher-level (almost 100%) inactivation of the highly expressed (5,000 mRNA molecules per cell) target hGH RNA (Table 1) was achieved by T7-dependent transcription in stable transfectants, which is most probably due to the higher level of expression. According to our calculations, the ratios of ribozyme to target RNA in our cellular assays were between 2:1 and 5:1 for pol III-dependent transcription and between 10:1 and 20:1 for T7-dependent transcription. In the former case we observed about a 50% reduction at the RNA level and 75% reduction at the protein level, whereas in the latter case we detected no hGH-specific RNA in some clones and found a 99.8% reduction at the protein level in a population of clones. This result clearly suggests that at least a 10-fold excess of ribozyme over the target RNA is required to achieve an almost complete inactivation of gene function.

Specificity and efficiency of ribozyme function. Bertrand et al. (5) asked whether ribozymes represent an improvement over antisense RNAs. Obviously, for both strategies an excess of the antisense molecule is required. Our direct comparison of selected ribozymes with the mutant version, which should function perfectly as an antisense molecule, clearly shows the difference. Very little effect of the mutant ribozyme was detected. It seems unlikely that the binding affinity of the mutant ribozyme differs from that of the functional one, but the length of the target-binding arms might be suboptimal for antisense function. Since other authors used much higher ratios of anti-

sense RNA or ribozyme to target RNA, our results point to the importance of using optimized ribozyme structures and target sites.

Different ribozymes for site E1 of the hGH mRNA with different lengths of the complementary region cleave the hGH RNA in vitro with a similar efficiency, but they appeared to have different cleavage efficiencies in vivo (Table 1). The degree of reduction of hGH expression achieved by different E1 ribozymes depends on the length of the complementary region. A reduction in the length of the flanking regions from 26 to 15 nt results in a fivefold increase of hGH inhibition if ribozymes are expressed by pol III expression of ribozymes in a transient-expression assay. This confirms the data of others (5, 16, 18, 23) showing that the turnover (k_{cat}) is slowed if the complementary region of the ribozyme extends beyond a certain length. If the ribozyme is in excess (500- to 1,000-fold) over the substrate RNA, the rate-limiting step is the cleavage reaction itself and not the ribozyme dissociation from the cleaved substrate (16, 17). The latter reaction would be important at lower ratios of ribozyme to target RNA. Our in vivo results (Table 1) obtained with selected ribozymes transcribed either by pol III or by T7 polymerase confirm that an excess of an optimal ribozyme is still required. However, for T7 transcripts the excess of ribozyme over hGH mRNA is only about 10:1. This level is sufficient to reduce the amount of detectable hGH protein to less than 99%, suggesting that several turnover reactions are carried out by the same molecules. More data applying optimal ribozymes to the inactivation of other genes expressed at very high or very low levels are required before we can draw a general conclusion about the excess of ribozyme over the target required to approach 100% inactivation of the target RNA in vivo. However, one cannot imagine that similarly effective inhibition would be obtained with antisense RNA only.

Prediction of cleavage sites. It is particularly striking that the cleavage sites experimentally detected within the hGH mRNA are all located within loops or single-stranded regions predicted by the computer program. On the one hand, this suggests that the model probably reflects the correct secondary structure of hGH RNA in these regions, and on the other hand, it confirms previous data obtained by others (26). These authors could show that ribozymes targeted to open stem-loop structures predicted by a computer model within the lactalbumin mRNA were more effective in reducing the level of RNA than were ribozymes targeted to a base-paired region. More data relating the results of computer-aided predictions of secondary structures of RNA to in vivo specificity and activity of ribozymes are required before general rules for the prediction of accessible ribozyme cleavage sites can be established.

Conclusion. Our results not only confirm the general idea that ribozymes are efficient tools for the inactivation of mRNA function but also provide, for the first time, a strategy for specific and efficient targeting of literally any target RNA. The principle is based on selection of accessible target sites by members of a ribozyme library and subsequent identification of the best-fitting ribozyme from the library. Although target site selection is carried out in vitro, there is a good chance that at least one of the ribozymes identified for a particular target site will also efficiently function in vivo. The examples presented here support this assumption. The use of our ribozyme library can circumvent expensive and time-consuming trial-and-error experiments in testing individual ribozymes designed for a particular target. A combination of target-selected ribozymes with the recently developed technique for intracellular colocalization of the ribozyme and its target (41) could be highly efficient in the inactivation of viral function, e.g., in the treatment of human immunodeficiency virus (36, 41, 49) or hepatitis virus infections. To this end, a new version of the library will be generated in which the random target-binding sequences flanking the hammerhead are only 7 nucleotides long on both sides. This will allow us to generate a representative collection of ribozymes.

ACKNOWLEDGMENTS

We thank Uta Fischer and Kordelia Hummel for technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (grant SFB344) and by the Fonds der Chemischen Industrie.

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